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Antibacterial effects of hydrogen peroxide and silver composition on selected pathogenic enterobacteriaceae

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ABSTRACT

Aims: Antibacterial effects of hydrogen peroxide and silver composition on selected pathogenic enterobacteriaceae was investigated in this study.

Materials and Methods: The efficacy of 30 ppb silver in 0.3% hydrogen peroxide solution for inactivation of selected Enterobacteriaceae, including *Escherichia coli, Proteus mirabilis,* and *Klebsiella pneumoniae* was assessed for 72 hours in a designated nutrient broth medium and steel surface. The bactericidal growth ability was determined for each bacterium genus by the conventional colony count method and turbidimetry via an optical density (OD) assay at 450 nm in a time interval of 24 hours.

Results: Suspensions of *K.pneumoniae*, and *P.mirabilis* showed a significant OD reduction at three 24-hour intervals (CI = 0.95; $P < 0.05$, for both), along with blocked growth in a designated broth medium during 24 to 48 hours of exposure. The disinfectant was also significantly efficient for inactivating of the mentioned bacteria on steel surfaces after a 15-minute time exposure (CI = 0.95; $P < 0.05$). For *E.coli*, the OD decreased slightly during the initial exposure time, but increased after 24 hours. Viable *E.coli* cells were proved by colonies grown on the plate. A qualitative surface decontamination test showed that three pathogenic bacteria were inactivated significantly after disinfectant exposure (CI = 0.95, $P < 0.05$).

Conclusions: In conclusion, a combination of hydrogen peroxide and silver ions was proposed as a strong disinfecting agent both in suspensions and on the surfaces against these three important human pathogens.

Key words: Disinfection, *E.coli*, hydrogen peroxide, *K. pneumonia, P. mirabilis*, silver

INTRODUCTION

Surface sterilization and water disinfection for drinking purposes are two major applications of disinfectants. At present, chlorine is the most popular disinfectant for water treatment.[1-4] Glutaraldehyde and peracetic acid are extensively used for sterilization of medical equipment and environmental surfaces.[5] However, some disadvantages, such as, formation of toxic disinfection by-products (DBPs) associated with chlorine,[6] mutagenic and carcinogenic effects of glutaraldehyde, and the high instability of peracetic acid,[7] have created doubts about their usage. To be an ideal disinfectant, an antimicrobial agent should have no residual toxicity, be safe for humans and animals, and be practically stable.[1,6,8]

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Two of the best disinfectants known until now are hydrogen peroxide (H_2O_2) and silver ions, and their strong bactericidal activities have been studied against three different bacterial genera. It has been reported that 30–100 ppm of H_2O_2 inactivate *E. coli* via DNA damage.[9] Hydrogen Peroxide Vapor (HPV) and its dry mist (DMHP(form also have bactericidal activity against mycobacterium tuberculosis, an important human pathogen,[10,11] *Clostridium difficile* spores, and its vegetative forms.[1,12] Moreover, the antibacterial effect, both on gram positive and gram negative bacteria, of silver in ionic or nanostructure forms has been established in the previous studies.[13] Subsequently,such materials have been proposed as water disinfectant agents.[13,14] Even as the disinfection potencies of several concentrations of Ag^+ and H_2O_2 have been investigated separately on different bacteria, just a few studies used a combination of these two disinfectants. It is reported that a combination of Silver and Hydrogen Peroxide (1:1000) shows a higher inhibiting potency on *E.coli* growth than each individual agent.[3,5] However, at present, some countries use various concentrations of H_2O_2 : Ag^+ for disinfecting drinking water; but the applicability and efficacy of these agents are questionable.[3,5,15,16] The aim of this study is to evaluate the antibacterial effects of a combination of 30 ppb Ag^+ in 0.3% hydrogen peroxide on *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*. These Gram-negative, straight rod, facultative anaerobic, oxidase-negative, and catalase-positive bacteria comprise of 80 to 95% of the clinical isolates and are also found in water supplies and the bio-films formed on the surfaces.[17]

MATERIALS AND METHODS

Disinfectant preparation

Stock solutions were 30% hydrogen peroxide (Merck) and 800 ppm silver ions from the $AgNO_3$ (Merck) combination. The treatment solution containing 0.3% H_2O_2 plus 30 ppb Ag^+ was freshly made in deionized water, according to Pedahzur R *et al*.,[3] with modifications obtained experimentally by the researchers. All the glassware was soaked in 10% nitric acid (Merck) overnight, for trace element decontamination, rinsed with deionized water, and sterilized in an autoclave before use.

Bacterial suspensions preparation

E. coli, *K. pneumoniae* and *P. mirabilis* were isolated from the hospital samples and confirmed by standard methods for diagnosis and differentiation of Gram negative pathogens according to the National Health Service (NHS) guidelines.[18] We designed a base medium containing peptone Broth (Gibco), which was diluted according to the user instructions, plus pure glucose powder (Sigma), with a concentration of 100 mg/dl; this base medium was used as the broth medium and was a candidate for an enriched aqueous environment. Primarily isolated colonies of bacteria, which were obtained from a hospital laboratory were sub-cultured on Eosin Methylene Blue (EMB) agar (Gibco) and Nutrient Agar (NA) (Gibco) and incubated for 24 hours, at 35°C, to obtain sufficient amounts of bacteria for the preparation of bacterial suspensions. After colony-forming unit (CFU) determination it was revealed that CFUs of *K. pneumoniae* were approximately 8.5×10^5 CFU/ml, *P. mirabilis* were 30.83×10^5 CFU/ml, and *E. coli* were 88.6×10^5 CFU/ml.

Experiment 1: Effect of H_2O_2 + Ag^+ combination on the growth property of bacteria in the suspension

A suspension of each bacterium was prepared in the base medium (peptone broth plus glucose), and te optical density (OD) of this suspension was adjusted to 0.1 – 0.2 using turbidimetry assays, at a wavelength of 450 nm, and by dilution of the suspension with a base medium. Each bacterial suspension was divided into 15 tubes; each tube contained 10 ml of each bacterial suspension, followed by an OD assay and bacterial culture on an EMB agar, to evaluate the bacterial counts before H_2O_2 + Ag + combination addition. Treatment solution was added to 10 tubes as test group in a way that the ultimate concentrations of disinfectant ingredients were 30 ppb silver and 0.3% H_2O_2. For the 5 remaining control tubes nothing was added.

Bacterial growth was assayed during three 24 hours intervals at 450nm spectrophotometrically as well as culturing and colony counting on EMB agar to determine colony forming units (CFU); all bacterial suspensions were kept at room temperature and in dark conditions during experimental period. In all cases firstly OD were measured then culturing were performed, so if any of tubes had high OD value, samples were diluted in sterilized phosphate buffer saline before culturing on EMB agar; so when numerating colonies to measure CFUs dilution coefficients were included.

Experiment 2: effect of H_2O_2 + Ag^+ combination on qualitative growth property of bacteria on hard surface (steel)

As it is shown in Figure 1, to determine the efficacy of H2O2+Ag+ combination on a contaminated surface, with selected bacteria, a laboratory steel-surface bench was divided into thirty 20 × 20 cm areas and sterilized with alcohol immersing and fire. The bacterial culture was performed to confirm this sterilization procedure. The divided surfaces were contaminated separately with a partially heavy suspension, containing CFUs/ml mentioned in the 'Bacterial suspensions preparation' section, of *E. coli*, *K. pneumoniae*, and *P.mirabilis*. The bacterial culture was repeated. Subsequently, using a swab soaked in H_2O_2+Ag+ combination solution, sterilization was performed, and the bacterial culture was repeated again after an interval of 15 minutes. The culture media were incubated at 37°C for 24 hours in a microbiological incubator (Memert). Note that in all steps sampling from the surfaces and subsequent culturing were done by sterilized swabs.

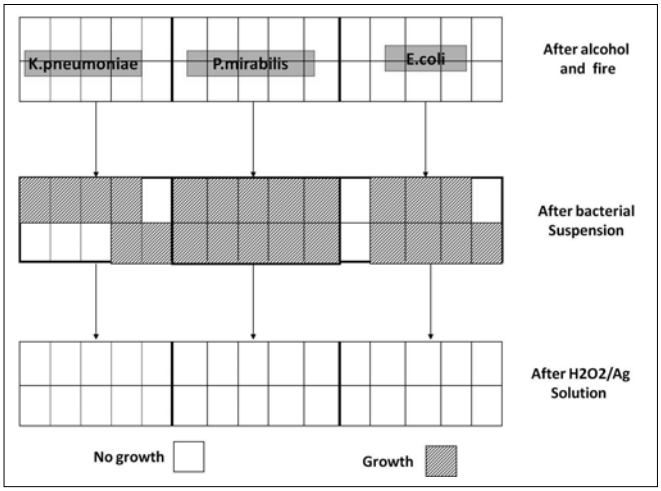

Figure 1: Schematic of the surface disinfection procedure and results of H_2O_2 + Ag^+ solution effects on *E. coli, K. pneumoniae,* and *P. mirabilis*

Statistical analysis

Data obtained from the cultures and optical density (OD) were analyzed using the SPSS software Ver. 11.5. We used the paired sample t-test, independent sample t-test, and the Chi-square/Fisher exact methods to compare the bacterial growth (mean CFU and OD) or to determine the disinfection efficacy.

RESULTS

Efficacy of H_2O_2+Ag^+ combination on bacterial growth in suspension

The efficacy of treatment solution against *K.pneumoniae* is shown in Figure 2. As it is revealed, the mean of OD in the test group shows a reduction during the first 24 hours, after addition of the H_2O_2+Ag^+ solution, while in the control group it increases rapidly over time; so comparison of the means shows a significant difference according to the statistical analysis (CI = 0.95, P = 0.001). For longer exposure times of more than 24 hours, the OD is virtually unchanged in the treatment group, but increases in the control tubes and a significant difference is obtained (CI = 0.95, P = 0.000). Also for ultimate hours of experiment there is a significant difference between the treatment and control groups (CI = 0.95, P = 0.000). For *K. pneumoniae*, the bacterial cultures of the treatment group were negative at 24 and 48 hours of H_2O_2 + Ag^+ solution addition. This means that treatment solution completely inhibits the growth of *K.pneumoniae* in the aqueous milieu.

Also the average values of OD and CFU for *P. mirabilis* were similar to *K. pneumoniae*, as shown in Figure 3. Comparison of the means of OD or CFU between the test and control groups at the start of the experiments were not significantly different (CI = 0.95; P = 0.146 and P = 0.371 for means OD and CFU, respectively). However, they were significantly different for the next 48 hours (CI = 0.95; P = 0.000 in the comparison both the mean of OD or CFU) and 72 hours (CI = 0.95; P = 0.000 for mean of OD). These results, similar to *K. pneumoniae*, showed that the treatment solution had blocked the growth ability of *P. mirabilis* in the aqueous medium.

Results for *E. coli* are somewhat different from those seen for *K.pneumoniae* and *P. mirabilis*; as is revealed in Figure 3. For *E. coli*, during the initial time of H_2O_2+Ag^+ solution addition, the mean OD is decreased slightly in the test group, whereas, it is increased in the control tubes (CI = 0.95; P = 0.000). Although the comparison of OD shows a significant difference, the colony counts of *E. coli* are not significantly different between the control and test groups of *E. coli* (CI = 0.95; P = 0.364). Post the initial 24 hours, both the mean OD and colony forming units (CFU) show an increment during 48 hours, but it is still significant in comparison to the control group (CI = 0.95; P = 0.000 for mean OD and P = 0.007 for mean CFU). As is shown in Figure 4, the mean OD of both the test and control groups are increased, but again they are still significantly lower than the control group (CI = 0.95; P = 0.000).

Efficacy of H_2O_2+Ag^+ combination on bacterial disinfection on hard surface (steel)

A summary of results is shown in Figure 1. By applying alcohol and fire, all surfaces were sterilized completely, which was confirmed by a culture on the EMB agar. After contamination of the surfaces by bacterial suspensions, 6, 10, and 7 squares were positive for *K.pneumoniae*, *P.mirabilis*, and *E.coli*, respectively. In the final step, treatment solution was applied and cultures were repeated. There was no positive result on EMB. Comparison of the bacterial growth before and after treatment with H_2O_2 + Ag^+ combination solution showed a significant difference (CI = 0.95; P = 0.008 for *E.coli*, P = 0.014 for *K. pneumoniae*, and P = 0.002 for *P. mirabilis*).

DISCUSSION

Here we have shown that 30 ppb silver in 0.3% hydrogen peroxide has bactericidal activity against *E. coli*, *K. pneumoniae*, and *P. mirabilis*. It was observed that *K.pneumoniae* and *P.mirabilis* responded to the treatment solution in a similar pattern; both of them were completely inactivated in the suspension, which was confirmed by no growth on the EMB agar and the partially unchanged OD during the 72-hour follow-up. However, results for *E. coli* were somewhat different; initially the OD of the *E. coli* cell suspension was decreased slightly, but it increased significantly at 48 hours exposure time, along with approximately unchanged CFUs. We supposed that the combination of silver and hydrogen peroxide damaged only a few percent of the *E. coli* cells and those that survived began to grow after 24 hours, probably just when H_2O_2 decomposition occurred and its concentrations was reduced, or this could have been due to the very high

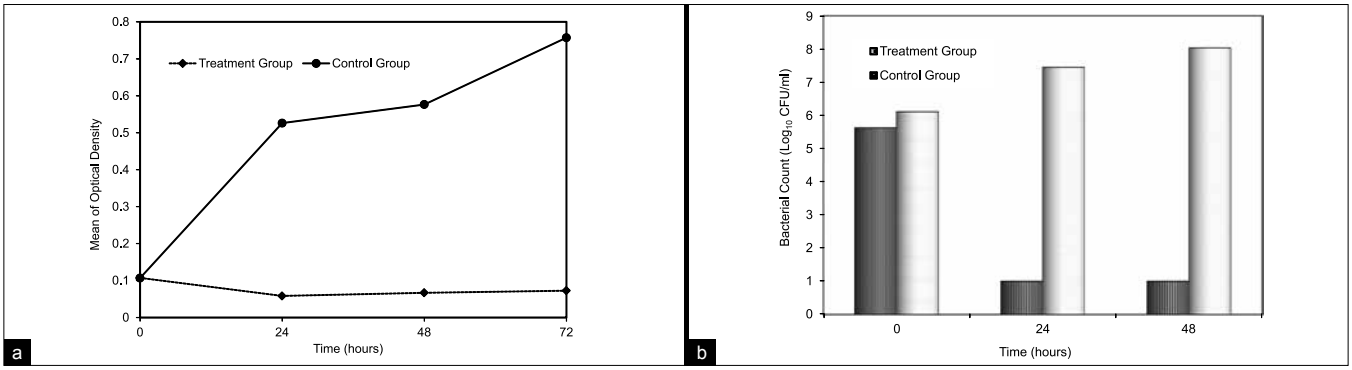

Figure 2: Effects of hydrogen peroxide and silver combined solution on *K.pneumoniae* suspension in terms of (a) Optical density and (b) Colony forming Units/ml during the shown periods

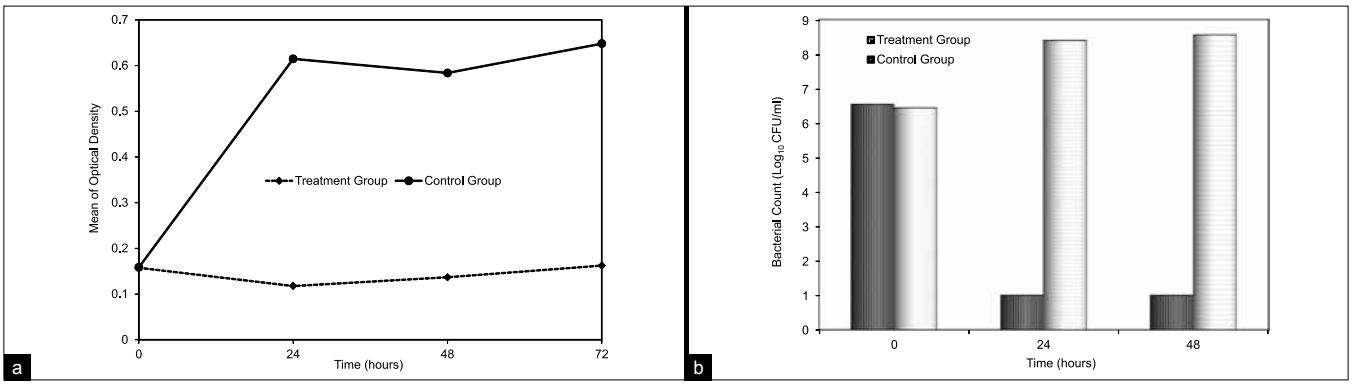

Figure 3: Effects of hydrogen peroxide and silver combined solution on *P. mirabilis* suspension in terms of (a) Optical density and (b) Colony forming units/ml during the shown periods

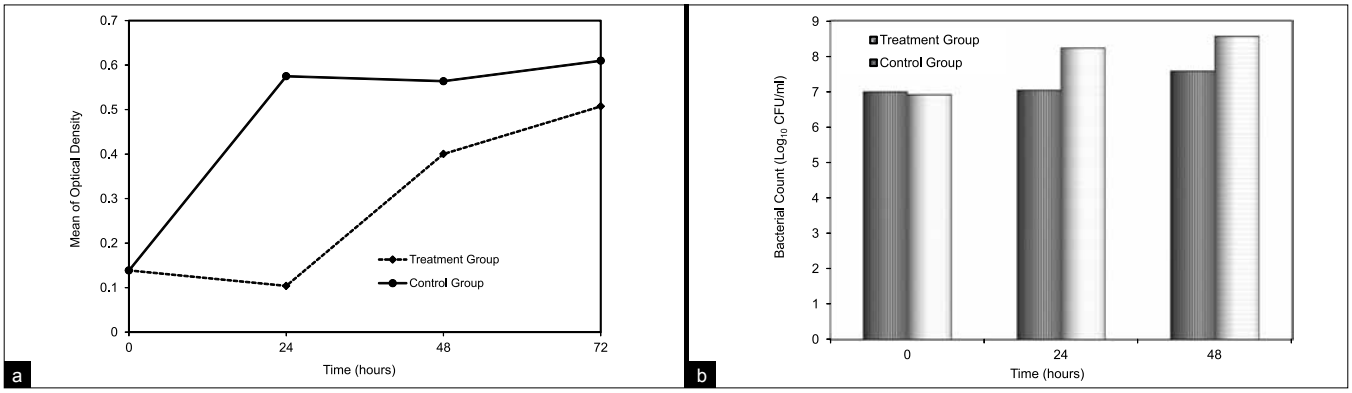

Figure 4: Effects of hydrogen peroxide and silver combined solution on *E.coli* suspension in terms of (a) Optical density, and (b) Colony forming units/ml during the shown periods

number of *E.coli* cells that were present in the suspension (see preparation of bacterial suspension under Material and Methods). However, these results also implied that low concentrations of silver, 30 ppb in this case, in the presence of a high number of E.coli cells did not inhibit bacterial growth, considering that the concentrations of H_2O_2 in the suspension could reduce significantly within 24 hours. Furthermore, there was evidence that *K. pneumoniae*,[19] *P. mirabilis*,[20] and *E. coli*[21,22] were all peroxidase-containing bacteria. Peroxidase and catalase enzymes were well known for decomposing hydrogen peroxide; so in the case of larger numbers of bacterial cells, as was revealed for *E. coli* and according to the CFU/ml, we hypothesized that more quantities of H_2O_2 were decomposed and the effective concentrations of this ingredient fell by that time. Pedahzur and coworkers reported that 60 minutes exposure to a combination of silver and hydrogen peroxide resulted in a 5 log reduction of *E. coli*;[3] however, their methodology, design of the study, and exposure time were different from our study. Furthermore, we had observed that all cultures of the three tested bacteria were negative within six hours of exposure time, for intervention, but positive for the control groups (data not shown). This finding was the same as that of Pedahzur *et al*.

Other studies have reported that low concentrations of H_2O_2 and Ag^+ reduce the total bacterial counts, including Enterobacteriaceae.[3,5,15,23,24] One study suggests that the interference of H_2O_2 in the Ag^+ efflux from the cell wall, as well as, interference of Ag^+ with H_2O_2 in cellular detoxification are possible modes of action of H_2O_2 and Ag^+ combination.[15] Barbut, F. and colleagues have shown that hydrogen peroxide dry-mist is significantly more efficient than sodium hypochlorite solution for disinfecting contaminated rooms with *Clostridium difficile* spores; they have reported that hydrogen peroxide dry-mist has reduced bacterial spores by about 91%, whereas, sodium hypochlorite has reduced only about 50% of the spores.[1] Also hydrogen peroxide dry-mist and vapor are highly potent for sterilizing of biosafety laboratories or equipment probably contaminated with *Mycobacterium tuberculosis*.[10,11] At least these two study results on the disinfection potential of hydrogen peroxide are in accordance with our results in both experiments 1 and 2, especially for hard surfaces. Surdeau, N and coworkers have reported that Oxsil 320N composed of hydrogen peroxide, acetic acid/peracetic acid, and silver is effective for deactivation of biofilms formed by planktonic bacteria on stainless steel surfaces. They have concluded that disinfection of such biofilms needs higher concentrations of tested disinfectant in comparison to the bacterial suspension.[25] Here we have worked on three pathogenic bacteria and the results are partially favorable for disinfecting the pathogens, both in suspensions and on stainless steel surfaces; furthermore, there are two major ingredients in our study, whereas, Oxsil 320N has at least three major ingredients. Although the research, design, and bacterial populations of Surdeau are somewhat different from our study, it seems that the current study has more promising results, because of the inactivation of the pathogenic bacteria by using a combination with lesser ingredients, which make the disinfectant very cost-effective. Armon, R *et al*. have reported that a combination of 30 ppm hydrogen peroxide and 30 ppb silver ions has been efficient in preventing biofilm formation in drinking or wastewater pipelines. Their results show that a combination of hydrogen peroxide and silver ions is more effective than either one alone.[26] However, we have tested higher concentrations of hydrogen peroxide because of higher colony forming coliforms in suspensions used in an aqueous model, or biofilms used on hard surfaces. As the tested bacteria show great adhesive properties on hard surfaces[27] the inactivation capability of H_2O_2 + Ag^+ combination will be worthwhile. In the case of biofilm formation, however, higher concentrations of the disinfectant may be necessary to achieve the same inactivation strength, as demonstrated in the literature.[25]

Anyway, we thought that the flaws and limitations of our study were: Determination of hydrogen peroxide concentration was not performed at each period and the efficiency of each ingredient alone, for deactivation of each bacterium, was not determined. Furthermore, our technique of sampling by swabs was practically inaccurate and imprecise; as is observed in Figure 1. After the contamination with heavily concentrated suspension of bacteria, there were still some negative squares, considering the bacterial growth on the EMB agar. However, the strong points of the current study were: Pathogenic bacteria isolated from the clinical samples were used and deactivated; and in this study we had designed an enriched broth medium that was suitable and applicable for the turbidimetry assay.

CONCLUSION

The current study demonstrates the strong disinfection effects of H_2O_2 + Ag^+ solution against three important human pathogens, which include *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Escherichia coli*. The authors believe that the use of H_2O_2 + Ag^+ combination may be realistic and applicable, because of high potency and cost-effectiveness of such a simple combination. However, further research needs to be designed for investigating the efficacy of such a combined disinfectant on other pathogen bacteria. On the other hand there are no reliable standardized techniques for monitoring bacterial responses to an intervention and this makes the study results difficult to interpret. We suggest that researchers work on the standardization of laboratory techniques. Also it is suggested that bacterial cultures be done along with OD assessment in *in-vitro* interventional laboratory trial studies, because each method has its interpretative value and one of them cannot be replaced for the other. Experimentally OD assessment is more precise, but not accurate, whereas, bacterial culture is more accurate, but less precise. Finally, it is necessary to design further studies on the applications of the mentioned disinfectant for the sterilization and disinfection of clinical/hospital surfaces especially on a broad spectrum.

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